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Review

Highlighting the role of Ras and Rap during *Dictyostelium* chemotaxis

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Abstract

Chemotaxis, the directional movement towards a chemical compound, is an essential property of many cells and has been linked to the development and progression of many diseases. Eukaryotic chemotaxis is a complex process involving gradient sensing, cell polarity, remodelling of the cytoskeleton and signal relay. Recent studies in the model organism *Dictyostelium discoideum* have shown that chemotaxis does not depend on a single molecular mechanism, but rather depends on several interconnecting pathways. Surprisingly, small G-proteins appear to play essential roles in all these pathways. This review will summarize the role of small G-proteins in *Dictyostelium*, particularly highlighting the function of the Ras subfamily in chemotaxis.

Keywords: *Dictyostelium*; Chemotaxis; Ras; Rap; Guanine exchange factor; GTP-binding protein

Contents

1. Introduction

Chemotaxis or directional movement towards a chemical compound is an essential property of many cells [1]. For example, chemotaxis is critical for the sourcing of nutrients by prokaryotes, the organization of the embryo in metazoa, the formation of multicellular structures in protozoa and the migration of lymphocytes during immune response [2–5]. Chemotaxis is also linked to the development and progression of many diseases including asthma, arthritis, arteriosclerosis, and cancers [6–8]. Therefore further insight into the molecular mechanism of chemotaxis is important for the understanding of many biological processes.

Research on eukaryotic chemotaxis has progressed substantially, mainly through the study of neutrophils or the amoeba *Dictyostelium discoideum* as model systems [1]. *Dictyostelium* is a free-living soil amoeba that feeds on bacteria. They chase bacteria by chemotaxing towards folic acid, which is secreted by the bacteria. Upon starvation cells undergo a tightly regulated developmental process. During development single cells undergo a drastic change in gene expression and start to secrete cAMP. Neighbouring cells respond by migrating toward the chemoattractant cAMP and by secreting cAMP themselves, resulting in multicellular fruiting bodies. Recently the assembly of the *Dictyostelium* genome was completed [9]. The 34 Mb

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genome contains many genes that are homologous to those in higher eukaryotes and are missing in other lower eukaryotes such as yeast [9]. As the mechanism of chemotaxis is essentially identical in all eukaryotes, *Dictyostelium* offers a genetically tractable model in which to study chemotaxis [1,10].

Chemotaxis is a complex cellular process involving a multitude of signalling pathways and molecules. Eukaryotic chemotaxis involves three distinct and separable processes: directional sensing, cellular motility and cell polarity [1,11]. The first step in directional sensing is the binding of the chemoattractant to cell-surface G-protein coupled receptors (GPCRs), which are characterized by seven membrane-spanning α-helices. Upon ligand binding, GPCRs undergo a conformational change that enables them to activate heterotrimeric G-protein; by exchanging the G-protein bound GDP to GTP. This exchange promotes dissociation of the three subunits as Gα-GTP and a Gβγ dimer, both of which can regulate a diverse set of downstream effectors. Due to the intrinsic Gα associated GTPase activity, GTP is hydrolysed to GDP, and the inactive heterotrimer is formed again [12–14].

In *Dictyostelium* four cAMP receptors have been identified (cAR1–cAR4). cAR1–4 are expressed sequentially throughout development and can all support chemotaxis. Since the types of cAMP receptor expressed sequentially during development have decreasing affinities, they are able to cope with an increase in extracellular cAMP concentration during the aggregation stage [15,16]. cAR2–4 have a relative low affinity for cAMP and are important during multicellular stage, whereas cAR1 has a high affinity for cAMP and is essential for signal transduction during early development and chemotaxis. *Dictyostelium* contains one G-protein β subunit and one Gγ subunit, which are both essential for chemotaxis. Of the 11 identified Gα subunits, Gα2 is coupled to the cAR1 receptor and most important for cAMP-mediated chemotaxis [17–19], Gα1 is coupled to a yet unidentified CMF receptor [20] and to cAR1 leading to inhibition of PLC activity [21], while Gα4 is most likely coupled to a yet unidentified receptors for folate [20,22]. Disruption of Gα2 results in cells that are unable to aggregate and do not respond to cAMP stimulation [18], a phenotype similar to strains deleted in cAR1 and Gβ [18,23,24], these phenotypes indicate that their function is essential for directional sensing and chemotaxis. Surprisingly, detailed analysis of the localisation and dissociation of these upstream components of the chemotactic signal-transduction machinery has revealed that cell polarization is established downstream of G-proteins. In cells exposed to a cAMP gradient, cAR1 is expressed uniformly on the plasma membrane [25], as are the G-protein subunits [26,27], while G-protein activation reflects the shallow external gradient of cAMP [26]. Subsequently the activated G-proteins convert the signal into the interior of the cell where they activate a complex network of signalling molecules, resulting in a gradient of cellular components. This gradient induces coordinated remodelling of the cytoskeleton and cell adhesion to the substratum, which leads to formation of new actin filaments in the front that induce the formation or stabilization of local pseudopodia, and acto-myosin filaments that inhibit pseudopod formation and retract the uropod. The final outcome is cellular movement up the chemoattractant gradient.

An important response in both the establishment of cell polarity and chemotaxis is the formation and accumulation of phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) at the leading edge [28–30]. Phosphoinositide 3-kinase (PI3K) produces $PI(3,4,5)P_3$ by phosphorylation of "$PI(4,5)P_2$", and phosphatase and tensin homologue deleted on chromosome ten (PTEN) converts $PI(3,4,5)P_3$ back to $PI(4,5)P_2$. Upon stimulation PI3K is rapidly translocated and activated at the leading edge, while PTEN is delocalised from the front to the lateral and posterior membrane [28–30]. By this reciprocal temporal and spatial regulation of PI3K and PTEN a $PI(3,4,5)P_3$ gradient is accomplished. The accumulation of $PI(3,4,5)P_3$ at the leading edge results in recruitment of $PI(3,4,5)P_3$ binding molecules and subsequent pseudopod extension [28,30,31]. Although, initial studies described an essential role for PI3K, recent studies have shown that chemotaxis does not depend on the $PI(3,4,5)P_3$ pathway [32–34]. Cells with disruption in all five genes encoding type I PI3Ks still exhibit normal chemotaxis, suggesting that PI $(3,4,5)P_3$ mediated signalling works in conjunction with one or more pathways to mediate chemotaxis [33]. Two studies identified phospholipaseA2 as a chemotactic pathways parallel to the PI3K pathway [32,34]. Chen et al. described that loss of the Ca^{2+}-independent phospholipaseA2 (iPLA2) homologue *plaA* results in cells that are hypersensitive to the PI3K inhibitor LY294002 and show dramatically reduced chemotaxis. Consistently, van Haastert et al. showed that inhibition of either PI3K or PLA2 has minor effects on chemotaxis, whereas inhibition of both enzymes inhibits chemotaxis nearly completely. The effects of inhibition of PI3K and PLA2 on chemotaxis are less severe at later stages of development, suggesting the presence of a third pathway in longer starved cells. Genetic studies suggest that this third pathway consists of a soluble guanylyl cyclase [35]. These data show that chemotaxis does not depend on a single molecular mechanism but depends on several interconnecting pathways.

Many studies have focused on the signal pathways downstream of the heterotrimeric G-proteins. Several of these studies have revealed that an early event in chemotaxis is the activation of small G-proteins. In this review we will focus on small G-proteins in *Dictyostelium*, particularly highlighting the important role of the Ras subfamily in chemotaxis.

2. Small G-proteins

Small GTPases are monomeric GTP-binding proteins, which function as molecular switches to control a wide variety of cellular functions. They switch between an inactive GDP-bound and active GTP-bound state (Fig. 1). Ras activity is regulated by guanine nucleotide exchange factors (GEFs) that catalyse the exchange of GDP for GTP, thereby activating the Ras protein. GTPase activating proteins (GAPs) stimulate an otherwise low intrinsic GTPase activity by many orders of magnitude, reverting the conformation back to the inactive GDP-bound form [36]. The eukaryotic small GTPase superfamily can be divided into five major subfamilies: Ras, Rho, Rab, Ran and Arf [37]. In the genome of *D. discoideum* an unusual large amount of small GTPases were identified. It contains a total of 119 genes encoding small GTPases, encompassing all five subfamilies [9,38]. Proteins of the Rab, Ran and Arf subfamilies are mostly implicated in the regulation of vesicular or nuclear transport,

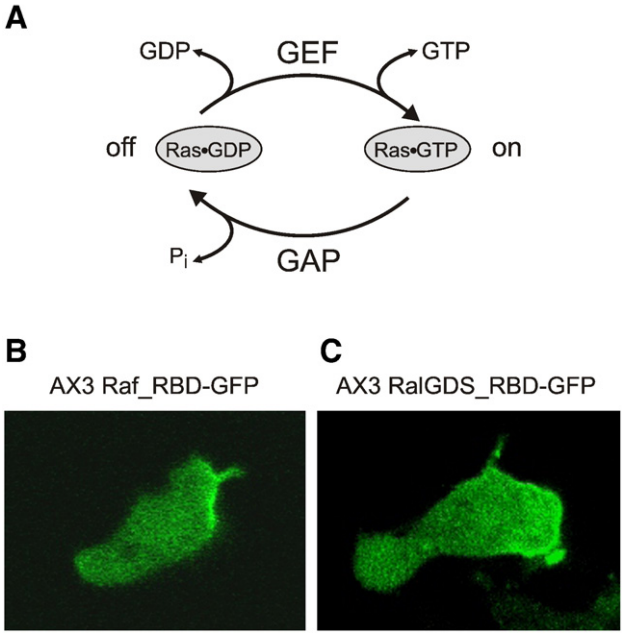

Fig. 1. *Ras signalling*. (A) The G-protein cycle. Small G-proteins switch between an inactive GDP-bound and active GTP-bound state. GEFs (guanine exchange factors) activate GDP-bound Ras by catalysing the exchange of GDP for GTP. Only in the active GTP-bound state, Ras can interact with downstream effectors. GAPs (GTPase activating proteins) catalyse the return to the inactive GDP-state, by stimulating an otherwise low intrinsic GTPase activity. (B) Ras activation at the leading edge of chemotaxing cells. Ras activation in vivo monitored by using GFP tagged RBD of Raf1 (Raf_RBD-GFP), which binds to and thus visualizes Ras-GTP levels and its localisation. (C) Rap1 activation at the leading edge of chemotaxing cells. In vivo Rap activation as visualized by GFP tagged Ras-binding domain (RBD) of RalGDS which binds to and thus monitors Rap1-GTP levels and its localisation.

while proteins of the Ras and Rho subfamilies are important for the transduction of extracellular signals [37]. Initially Rho GTPases were implicated in actin-regulated processes such as morphogenesis, membrane trafficking and motility; however more recently Rho has been shown to act in numerous other pathway as well, including, cytokinesis, apoptosis, tumorigenesis and cell cycle progression [39,40]. During chemotaxis, Rho GTPases are essential for the organization of myosin and remodelling of the actin cytoskeleton. Subfamily Ras GTPases are involved in regulating several cytoplasmic signalling cascades including cell growth, proliferation, differentiation and survival [37]. Recent studies, mostly performed in *Dictyostelium*, have shown the importance of Ras subfamily members in eukaryotic chemotaxis. During chemotaxis, Ras proteins are key intermediates in directional sensing, cellular motility and cell polarity [41].

3. *Dictyostelium* Ras GTPases

Because of their critical role in human oncogenesis Ras (*Ras sarcoma*) proteins have been the subject of intensive research [42]. The *Dictyostelium* Ras GTPase subfamily comprises 15 proteins; 11 Ras, 3 Rap and one Rheb related protein [38]. Thus far six Ras subfamily members have been characterized, and they all appear to have important roles in cell physiology (Table 1). The first described Ras gene, *rasD*, was identified as a gene up regulated in the prestalk population [43]. Genetic studies have linked RasD to the regulation of both phototaxis and thermotaxis of multicellular slugs [44]. Localisation and mutant studies suggest an important role for RasB in progression through the cell cycle. RasB is localised in the nucleus except during mitosis/cytokinesis. Mutants carrying a disruption of the *rasB* gene are unstable and exhibit severe growth defects; cells expressing the dominant active RasB (G12T) show abnormal cytokinesis [45]. Several attempts to generate stable *rasB*-null cells failed, suggesting an essential role for RasB [38,45]. Disruption of *rasS* results in cells that have defective growth, impaired endocytosis, are highly polarized and migrate threefold more rapidly than wild-type cells, suggesting inappropriate control of the actin cytoskeleton [46,47]. RasC and RasG are the best characterized *Dictyostelium* Ras proteins, and both are activated in response to cAMP [48]. RasG and RasC have both unique and overlapping functions. RasG regulates cell motility and the cytoskeleton during the vegetative state. Both RasC and RasG are involved in the regulation of the cAMP relay and cAMP-mediated chemotaxis [49–51]. The only thus far characterized *Dictyostelium* Rap subfamily member, Rap1, is involved in

Table 1
Overview of the *Dictyostelium* Ras subfamily

	Effect of disruption	Regulators	Effectors	Function
rasB	Not stable			Cell cycle/mitosis
rasC	Reduced ACA activation, developmental defects	GEFA	ACA	cAMP relay, chemotaxis
rasD	Slug phototaxis and thermotaxis defects			Phototaxis, thermotaxis
rasG	Growth, developmental and chemotaxis defects	GEFR	PI3K, RIP3, GC	Motility, cytoskeleton, chemotaxis
rasS	Endocytosis, pinocytosis and motility defects	GEFB		Balance between feeding and movement
rasU				
rasV				
rasW				
rasX	No phenotype			
rasY	No phenotype			
rasZ	No phenotype			
rapA	Not viable	GbpD, RapGAPA	Phg2	Cell polarity, adhesion, cytoskeleton and motility
rapB				
rapC				
rheb				

proliferation, growth, adhesion, and regulation of the cytoskeleton [52–54]. Multiple attempts to generate *rapA*-null cells have failed and antisense *rapA* mRNA resulted in cells with defects in growth and viability, suggesting an essential role for RapA in *Dictyostelium* [52].

4. Ras signalling at the leading edge

Several studies in *Dictyostelium* have shown the important role of Ras in cAMP-mediated chemotaxis. Upon cAMP stimulation both RasC and RasG are rapidly activated [48]. Both in vegetative cells and in stimulated cells, GFP fused Ras proteins are uniformly distributed along the membrane [55], indicating that the localisation of Ras does not change upon stimulation. Ras activation in vivo can be visualized by a GFP tagged Ras-binding domain (RBD) of Raf1 (Raf_RBD-GFP), which binds to and thus monitors Ras-GTP levels and its localisation [55,56]. Raf_RBD-GFP, which is mainly localised in the cytosol of non-stimulated cells, rapidly translocates to the membrane upon global stimulation [55]. When expressed in chemotaxing cells, Raf_RBD-GFP exclusively localised to the leading edge (Fig. 1B). Therefore, although Ras proteins are uniformly distributed along the plasma membrane in chemotactic cells, Ras activation primarily takes place at the leading edge [55]. More evidence for the important role for Ras in chemotaxis came from genetic studies.

Disruption of both *rasC* and *rasG* results in the total loss of cAMP-mediated signalling; cells fail to aggregate under all conditions, have no cAMP relay and fail to exhibit chemotaxis towards cAMP [49]. These data suggest that all cAMP signal transduction in early development is partitioned between pathways that use either RasG or RasC (Fig. 2). The exact mechanism of RasC and RasG activation is not clear, but apparently requires functional heterotrimeric G-protein since cAMP-stimulated Ras activation is absent in mutants lacking Gβ or Gα2 [48,55]. Since Ras is still activated in cells treated with the actin-polymerisation inhibitor Latrunclin A, a functional cytoskeleton is not essential for Ras signalling. Loss of PI3K activity, either through gene knockout or use of the PI3K inhibitor LY294002, has only mild effect on Ras activation, indicating that also $PI(3,4,5)P_3$ formation is not necessary for Ras signalling. Ras activation is catalysed by GEF proteins. *Dictyostelium* contains 25 genes encoding putative Ras-GEFs, five of them have been characterized to some extent [57]. Multiple studies have indicated the important role of Aimless (AleA or GEFA) in chemotaxis. Disruption of *gefA* results in cells defective in aggregation, mainly caused by loss of the cAMP relay, but cells also have a chemotaxis defect [58]. The *gefA*-null phenotype shares some characteristics with *rasC*-null cells and some with *rasG*-null cells [49,50,55], so it remained long unclear whether the main target of GEFA is RasC or RasG. However, a recent study by Kae et al. suggests that GEFA is specifically activating RasC, while *Dictyostelium* GEFR is involved in activation of RasG [48].

The best-studied downstream effector of Ras is PI3K. Upon cAMP stimulation PI3K is recruited to the leading edge and phosphorylates $PI(4,5)P_2$ to form $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ is a very strong inducer of pseudopod extension and important for mediating directional sensing [28–30]. PI3K consists of an N-terminal membrane-targeting region, a RBD, a C2 lipid binding domain (PI3Ka) and a PI3K catalytic domain (PI3Kc). The RBD domains of PI3K1 and PI3K2 are required for chemoattractant-stimulated PI3K activity, but not PI3K translocation [28]. RBD domains have an ubiquity fold and interact tightly only with the GTP-bound but not with the GDP-bound conformation of Ras-like proteins [59]. Previous studies have shown that the RBD of PI3K1 and PI3K2 interact weakly with RasD and strongly with RasC and RasG [28,48]. More evidence for a role of Ras in $PI(3,4,5)P_3$ signalling comes from several genetic studies: *rasC*-null and *rasG*-null cells have a strongly reduced Act/PKB response, a downstream effectors of PI3K, while cAMP-mediated $PI(3,4,5)P_3$ production is completely

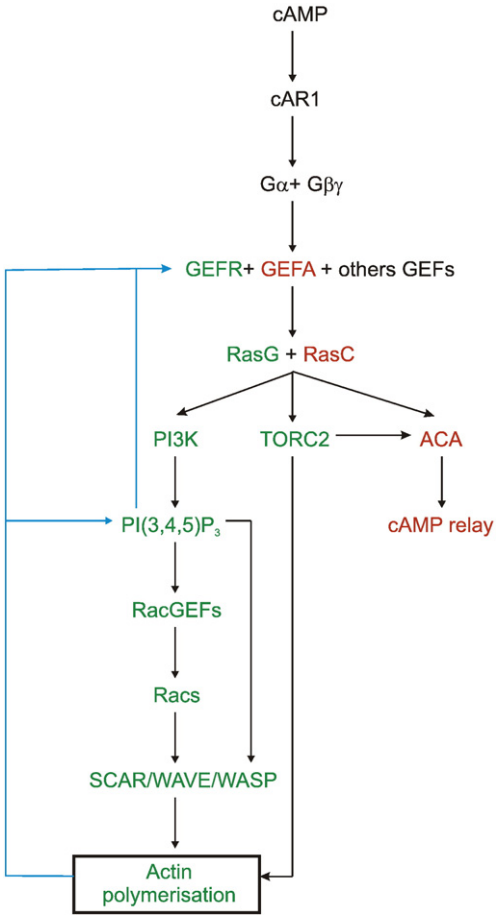

Fig. 2. *Ras signalling at the leading edge*. Binding of cAMP to the cAR1 receptor results through heterotrimeric G-protein signalling to the rapid activation of GEFs and subsequently Ras at the leading edge. cAMP signal transduction in early development is partitioned between pathways that use either RasG (depicted in green) or RasC (depicted in red). RasG and RasC seem to have both unique and overlapping functions. RasG (indicated in green) is more important for regulating PIP3 signalling and TORC2 activity and subsequently essential for cell polarity and actin polymerisation at the leading edge. RasC on the other hand is more important for ACA activity and hence cAMP relay. Although, Ras is activated independent of PI3K or actin, a positive feedback loop (indicated in blue) involving $PI(3,4,5)P_3$ mediated actin polymerisation, recruitment of additional PI3K to the leading edge, and Ras activation is important for amplifying the initial signal and stabilizing the leading edge.

absent in *rasC*/*rasG*-null [49]. Furthermore, over expression of dominant active RasG12V leads to prolonged accumulation of $PI(3,4,5)P_3$ [55].

Although the initial Ras response is independent of PI3K and actin polymerisation, some studies have suggested the presence of a positive feedback loop between F-actin, PI3K and Ras [55]. In vivo studies, using Raf_RBD-GFP of Raf1 to monitor Ras activation, showed reduced Ras activation at the leading edge of cells treated with the actin-polymerisation inhibitor Latrunclin A or PI3K inhibitor LY294002. Furthermore, randomly moving *pten*-null cells show multiple and broad patches of active Ras and $PI(3,4,5)P_3$ from which multiple pseudopodia are extended. After treatment with Latrunclin A or LY294002, both the spontaneous Ras activation and $PI(3,4,5)P_3$ accumulation in *pten*-null cells are completely abolished. These results indicate that both PI3K and actin can regulate Ras activation. Since Ras activation is essential for PI3K activation, the presence of a positive feedback loop was suggested: Upon cAMP stimulation, Ras is rapidly activated at the leading edge, independent of PI3K or actin. Ras activation results in the activation of PI3K, and subsequently the production of $PI(3,4,5)P_3$ at the leading edge. A positive feedback loop involving $PI(3,4,5)P_3$ mediated actin polymerisation, recruitment of additional PI3K to the leading edge, and Ras activation is important for amplifying the initial signal and stabilizing the leading edge [55,60].

5. Ras signalling and regulation of cellular motility

The establishment of an intracellular gradient leads to major changes in the cytoskeleton; actin polymerisation occurs at the leading edge of the cell, while acto-myosin filaments are formed at the rear and side of the cell. The new actin filaments establish new adhesion sites in the front and induce the formation or stabilization of local pseudopodia. The acto-myosin filaments inhibit pseudopod formation in the rear and retract the uropod. In this way coordinated cell movement is achieved. Actin polymerisation is mediated by the Arp2/3 complex which binds to the sides of pre-existing actin filaments and produces a complex network of branched filaments that leads to pseudopod extension [61]. The regulation of this process involves several groups of proteins, including the Ras and Rho subfamily of small G-proteins, WASP (Wiskott–Aldrich syndrome protein), and WAVE/Scar [62,63].

Regulation of the cytoskeleton at the sites and in the back of the cell is as important as its regulation at the leading edge. In moving cells myosin-II filaments are formed at the cortex in the back and at the sites of cells, thereby preventing the formation of lateral pseudopods and providing the power to retract the uropod [64]. In *Dictyostelium*, myosin assembly seems to be strictly dependent on the phosphorylation state of the myosin heavy chain (MHC) [65,66]. Phosphorylation by MHCKs inhibits filament formation [67,68], whereas dephosphorylation by protein phosphatase 2A is essential for myosin disassembly [69]. Phosphorylation of the myosin light chain (MLC) by MLCKs, promotes myosin motor activity, which is important for supplying contractile force to retract the rear of the cell [65,66]. In Fig. 3, a schematic overview of the pathways

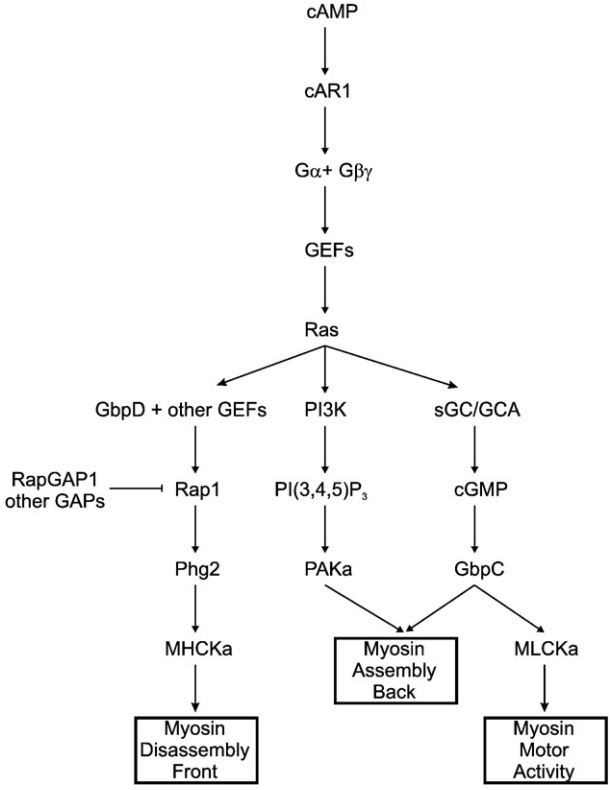

Fig. 3. *Ras regulation of myosin-II filaments in chemotaxis*. Rap1 regulates leading edge formation by controlling myosin formation at the leading edge. Upon cAMP stimulation Rap is rapidly activated at the leading edge. Rap-GTP binds and activates Phg2, which directly or indirectly phosphorylates MHCKa, thereby inhibiting myosin filament formation at the front. In the contrary myosin-II filaments are formed at the cortex in the back of moving cells, thereby preventing the formation of lateral pseudopodia and providing the power to retract the uropod. Myosin assembly at the back is mainly regulated by PAKa and the cGMP pathway, both of which are activated by Ras. cAMP-mediated Ras activations lead, through a yet unknown mechanism, in the rapid activation of the guanylyl cyclases, sGC (soluble guanylate cyclase) and GCA (guanylyl cyclase A). The resulting cGMP activates GbpC which mediates myosin in two ways. It regulates myosin assembly at the back of the cell and secondly it regulates MLCKa phosphorylation, which promotes myosin motor activity, thereby enhancing the retraction force of myosin filaments causing the uropod to retract.

regulating myosin-II filament formation in *Dictyostelium* is depicted. Several studies have shown that cGMP is the key regulator of myosin formation [70]; cells lacking cGMP formation have an impaired recruitment of myosin-II to the cytoskeleton, and cells with elevated levels of cGMP have increased activation of myosin-light-chain kinase A (MLCKa) and subsequently an increased myosin motor activity.

cGMP is rapidly produced in response to cAMP by the enzymatic activity of two guanylyl cyclases and its degradation is performed by three phosphodiesterases [71,72]. Thus far only one downstream target of cGMP has been identified, the high-affinity cGMP-binding protein GbpC [73]. GbpC belongs to a new group of the GTPase superfamily, termed Roc (Ras of complex proteins), and contains leucine-rich repeats, Roc small GTPase, kinase, GEF and cyclic nucleotide binding domains [73,74]. Mutants lacking cGMP or GbpC show greatly reduced MLCKa activation and subsequently reduced RLC phosphorylation. Probably, cGMP binds to GbpC, thereby increasing its

kinase activity, which results in phosphorylation of MLCKa either directly or via an intermediate kinase.

Several Ras subfamily members have been linked to the activation of the cGMP pathway. Cells lacking *rasC* or *rasG* have a reduced cGMP response, while in the *rasC*/*rasG*-null mutant barely any response could be detected, indicating that both RasC and RasG are important regulators of the cGMP pathway [49]. Consistently, also mutants lacking *gefA* or the Ras effector *rip3* have a strongly reduced cAMP-mediated cGMP response [75]. Also Rap1 is linked to the cGMP pathway; cells depleted in Rap1 have a reduced cGMP response after osmotic shock, while cells expressing constitutively active Rap1(G12V) show an enhanced response [52].

Dictyostelium Rap1 is rapidly activated at the leading edge of chemotaxing cells (Fig. 1C), however with slower kinetics as those of Ras [76]. Rap1 is an important regulator of cytoskeletal rearrangement, phagocytosis, adhesion and chemotaxis [52–54]. Cells expressing dominant negative Rap(S17N) have a more polar elongated cell morphology and are less adhesive compared to wild-type cells, whereas cells expressing constitutive active Rap are flat, extend many substrate-attached pseudopodia, are highly adhesive and have impaired chemotaxis [53,76,77]. Consistently, a strikingly similar phenotype was observed in cells overexpressing the *Dictyostelium* RapGEF GbpD [78,79]. *Dictyostelium* GbpD was previously identified as putative cGMP-binding proteins. GbpD contains a CDC25-homology domain responsible for mediating GEF activity, a Ras exchange motif (REM) domain, a GRAM (glucosyltransferases, Rab-like GTPase activators, myotubalarins) domain and two cyclic nucleotide binding domains, that appear not to bind cAMP or cGMP [73]. Cells lacking GbpD display improved chemotaxis and are hyperpolar, because cells make very few lateral pseudopodia, whereas the leading edge is continuously remodelled [78,80]. Overexpression of GbpD results in severely reduced chemotaxis. Cells extend many bifurcated and lateral pseudopodia, resulting in the absence of a leading edge. Furthermore, cells are flat and adhesive owing to an increased number of substrate-attached pseudopodia [78]. Although GbpD contains two cNB domains, it is not regulated by cAMP or cGMP: Radioactive cyclic nucleotide binding assays with cells overexpressing GbpD or from *Eschericha coli* purified protein revealed no detectable binding of cAMP or cGMP [80]. In support of these data, the strong GbpD^{OE} phenotype is independent of the presence of cAMP/cGMP [78]. Since Rap1 appears to be essential in *Dictyostelium*, whereas disruption of *gbpD* is not lethal and *gbpD*-null cells still contain active Rap [79], there must be at least one additional GEF protein for Rap. Besides multiple Rap specific GEFs, as many as nine putative *Dictyostelium rapGAP*s have been identified. Of these *rapGAP*s, which catalyse the inactivation of Rap, thus far only *rapGAP1* has been characterized [81]. RapGap1 localises at the leading edge with slightly delayed properties as Rap1-GTP, *rapGAP1*-null cells have increased and extended Rap1-GTP activation and a phenotype identical to cells overexpressing RapG12V or GbpD. These data underline the important role of Rap in cell adhesion, cell polarity and chemotaxis [76,79,81].

The mechanism by which Rap regulates its functions is not completely understood, however recent studies provided evidence that Rap1 is involved in inhibition of myosin-II at the leading edge of *Dictyostelium* cells [76]. Cells expressing RapG12V exhibit strongly reduced cAMP-mediated myosin assembly and are unable to control cell contraction, a phenotype similar to cells lacking myosin-II [52,66,76]. In a screen for downstream targets of GbpD/Rap, the serine/threonine kinase Phg2 was identified as the first *Dictyostelium* Rap effector [79]. Like many of the mammalian Rap effectors, Phg2 contains a putative RA domain [82]. By yeast two-hybrid and guanine nucleotide dissociation measurements it was shown that the RA domain of Phg2 preferentially binds Rap1-GTP over Ras-GTP [79,82]. In the contrary to overexpression of GbpD or RapG12V in wild-type cells, overexpression of GbpD or RapG12V in *phg2*-null does not result in increased substrate attachment, indicating that Phg2 is essential for Rap1 mediated adhesion [79,82]. Upon cAMP stimulation Phg2 rapidly localises to the leading edge, extending weakly to the posterior sides, a temporal and spatial localisation similar to Rap1-GTP [76]. The RA domain of Phg2 is not necessary for its translocation but is essential for activity [76]. Disruption of *phg2* results in impaired chemotaxis, cells have a broad leading edge, extend multiple pseudopodia and move slowly [76]. Further study revealed that *phg2*-null cells have an increased amount of myosin-II and Phg2 is essential for efficient myosin-II phosphorylation in vitro [76]. These results suggest that Rap1 mediates at least partly its functions by controlling myosin-II assembly and disassembly through Phg2.

Another protein involved in regulation of myosin assembly is the p21-activated kinase PAKa; *pakA*-null cells, like cells deficient in cGMP production, fail to induce cAMP-mediated myosin association with the cell cortex [83]. The mechanism by which PAKa regulates myosin assembly is unknown, but does not involve direct phosphorylation of MHC. In a recent study it was shown that PI3K and PKB/Akt mediate both the localisation and activity of PAKa, thereby revealing an interesting link between the PI(3,4,5)P_3 pathway and regulation of myosin assembly [84]. Therefore PI(3,4,5)P_3 formation is important for both the regulation of pseudopod formation at the leading edge, as well for regulation of myosin assembly at the back of the cell.

6. Role of Ras in signal relay

For proper aggregation of *Dictyostelium* cells, cAMP relay, the process by which the signal is passed throughout the cell population, is essential [85]. As mentioned above, upon starvation *Dictyostelium* cells enter the developmental stage eventually resulting in the formation of multicellular fruiting bodies [86]. Starved *Dictyostelium* cells produce and secrete cAMP, which induces its own production by binding to and activation of the cAR1 receptor, resulting in the activation of ACA (adenylyl cyclase for aggregation) and cAMP production. Cells lacking ACA are devoid of chemoattractant-mediated cAMP production and therefore not able to aggregation [85]. Activity of ACA is regulated by Gβγ and needs in addition two novel cytosolic regulators: cytosolic regulator of adenylyl

cyclase (CRAC) and TORC2 [75,87–90]. Upon cAMP stimulation CRAC transiently translocates to the leading edge, by binding to PI(3,4,5)P_3 through its PH domain. Localisation of CRAC at the leading edge is required for proper activation of ACA [91]. Since CRAC localisation depends on PI(3,4,5)P_3 and PI(3,4,5)P_3 formation depends on Ras activation, Ras indirectly regulates ACA activity. Consistently, cAMP-mediated ACA activation is reduced in *pi3k*-null cells and *rasG*-null cells, further reduced in *rasC*-null cells and almost absent in *rasC*/*rasG*-null [49,92]. *rasC*-null and *gefA*-null cells are unable to aggregate, a defect which can be recovered by exogenous application of cAMP pulses [58,93]. Overexpression of CRAC cannot rescue the *gefA*-null phenotype, indicating that it is not caused by decreased CRAC activity [58]. Therefore it seems that the GEFA/RasC pathway activates a pathway parallel to CRAC that is essential for ACA activity.

Ras also regulates ACA activity through TORC2. The *Dictyostelium* TORC2 complex is formed by Pianissimo (Pia), RIP3 and LST8 [89]. Similar to *crac*-null cells, cells lacking *pia*, *rip3*, or *lst8* have an severely impaired cAMP response and are unable to spontaneously aggregate [75,87,89]. Like for *rasC*-null and *gefA*-null, exogenous pulsing with cAMP partly rescues the *rip3*-null phenotype, but not the aggregation defects of *pia*-null and *lst8*-null cells [89]. RIP3 was identified in a screen for proteins that interact with *Dictyostelium* Ras proteins and contains like many Ras effectors an RBD [75]. RIP3 carrying point mutations that disrupt binding to Ras-GTP is unable to rescue the phenotype of *rip3*-null cells, indicating that Ras is an essential regulator of TORC2 function [89]. Since RasC seems to be the primary Ras protein regulating ACA activity and *rasC*-null and *rip3*-null cells have similar defects, it was suggested that RasC might regulate TORC2 activity [49,75,89]. However, RIP3 interacts better with RasG than with other *Dictyostelium* Ras proteins in yeast two-hybrid assays, indicating that RasG might also be involved in regulation of TORC2 and ACA activity [75]. In addition to its putative role in ACA activation, evidence has been obtained suggesting that RasG is also a negative regulator of ACA activity. Cells expressing constitutively activated RasG (G12T) are defective in cAMP production and unable to aggregate [94]. Overexpression of constitutively activated RasG (G12T) severely inhibits ERK2 activation in response to both cAMP and folic acid [95], a response that is essential for ACA activation [96,97].

Although the pathways regulating ACA activity have only been partly characterized, Ras proteins are clearly important regulators of the cAMP relay.

7. Conclusion

Chemotaxis is a complex process involving gradient sensing, cell polarity remodelling of the cytoskeleton and signal relay. Surprisingly, Ras proteins appear to play essential regulatory roles in all these processes. Although knowledge about the function of Ras proteins has progressed quickly over the past few years, still many questions have to be addressed. So far only six Ras proteins have been characterized, only few Ras effectors have been identified and the temporal and spatial modulation of Ras activity through GEFs and GAPs is not well understood. Since Ras proteins are key mediators of chemotaxis, addressing these questions will greatly contribute to the understanding of chemotaxis.

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